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The scavenging of reactive oxygen species and the potential for cell protection by functionalized fullerene materials

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ABSTRACT

We demonstrated that three different types of water-soluble fullerenes materials can intercept all of the major physiologically relevant ROS. $C_{60}(C(COOH)_2)_2$, $C_{60}(OH)_{22}$, and $Gd@C_{82}(OH)_{22}$ can protect cells against H_2O_2-induced oxidative damage, stabilize the mitochondrial membrane potential and reduce intracellular ROS production with the following relative potencies: $Gd@C_{82}(OH)_{22} \geq C_{60}(OH)_{22} > C_{60}(C(COOH)_2)_2$. Consistent with their cytoprotective abilities, these derivatives can scavenge the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH), and the reactive oxygen species (ROS) superoxide radical anion ($O_2^{\bullet-}$), singlet oxygen, and hydroxyl radical ($HO^{\bullet}$), and can also efficiently inhibit lipid peroxidation *in vitro*. The observed differences in free radical-scavenging capabilities support the hypothesis that both chemical properties, such as surface chemistry induced differences in electron affinity, and physical properties, such as degree of aggregation, influence the biological and biomedical activities of functionalized fullerenes. This represents the first report that different types of fullerene derivatives can scavenge all physiologically relevant ROS. The role of oxidative stress and damage in the etiology and progression of many diseases suggests that these fullerene derivatives may be valuable *in vivo* cytoprotective and therapeutic agents.

1. Introduction

A large number of *in vitro* and *in vivo* studies suggest that oxidative stress is linked to either the primary or secondary mechanisms of progression for many acute and chronic diseases. As mediators of oxidative stress, reactive oxygen species (ROS), which include superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and hydrogen peroxide, have been implicated in the etiology of several human diseases, including amyotrophic lateral sclerosis, arthritis, cancer, cardiovascular disease, and a number of neurodegenerative disorders [1,2]. Cellular targets of ROS include DNA, proteins and lipids. Damage to these cellular targets has been associated with aging and several human diseases including cancer, athereosclerosis, ischemia, inflammation, and liver injury [3–5]. In addition, it has been determined that compared to normal cells, cancer cells are under increased oxidative stress, which is associated with increased generation of ROS and can result in stimulation of cellular proliferation, mutations, and genetic instability [6–8]. Thus, chemical species that potently scavenge ROS may be of great significance in biomedicine both for maintaining health and for use in cancer chemotherapy.

It is well established that fullerenes and their derivatives possess a unique capacity for scavenging ROS [9–11]. Because underivatized fullerenes are insoluble in water and biological systems, hydroxylated and other derivatized fullerenes have been utilized due to their increased water solubility and resulting increase in payload of ROS-scavening activity to target cells and tissues. Demonstrated protective effects of water-soluble fullerene derivatives include reduction of injury on ischemic reperfusion of the intestine [10], a decrease in numbers of cells undergoing apoptosis [11], reduction in free radical levels in organ perfusate [12], and neuroprotective effects [13].

Hydroxylated fullerenes (fullerenols) and malonic acid-substituted fullerenes (carboxyfullerenes) are two major groups in

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water-soluble fullerene materials, which were found to possess biological significance as free radical scavengers [9–15]. For example, Dugan et al. [14,15] demonstrated that carboxylic acid-substituted C_{60} derivatives had potent ROS-scavenging activity. These fullerene derivatives prevented apoptosis of cultured cortical neurons induced by exposure to N-methyl D-aspartate (NMDA)-agonists, protected the nigrostriatal dopaminergic system from iron-induced oxidative injury, and showed effective neuroprotective antioxidant activity *in vitro* and *in vivo*. Fullerenols have also been demonstrated to be particularly valuable candidates for use as free radical scavengers or water-soluble antioxidants in biological systems [16].

Endohedral metallofullerenes, i.e. compounds in which a fullerene encapsulates a metal atom(s), have shown great promise for use in biomedical science. Although C_{60} has been the most commonly studied fullerene in biological systems, few endohedral materials have been synthesized using C_{60} as a cage molecule because of the limited interior volume of C_{60}. Therefore, most endohedral metallofullerenes are synthesized using C_{82} or higher molecular weight fullerenes, and many derivatives of C_{82} fullerenes have been synthesized in our laboratory. Gd@C_{82} is one of the most important molecules in the metallofullerene family [17]. Gadolinium endohedral metallofullerenol (e.g., Gd@$C_{82}(OH)_{22}$) is a functionalized fullerene with gadolinium, a transition metal in the lanthanide family, trapped inside the C_{82} fullerene cage. We have previously reported that the chemical and physical properties of gadolinium endohedral metallofullerenols are dependent on the number and position of the hydroxyl groups on the fullerene cage [17]. These results demonstrated that modifying the outer cage of Gd@C_{82} with a number of hydroxyl groups tunes the electronic properties of the inner metal atom as well as the electron density and polarizability of the electrons at the fullerene's surface.

Gadolinium endohedral metallofullerenols were originally designed as magnetic resonance imaging (MRI) contrast agents for biomedical imaging [18]. These materials have additionally attracted attention due to their potential use in chemotherapy [18,19]. We have recently reported that aggregates of Gd@$C_{82}(OH)_{22}$, i.e. Gd@$C_{82}(OH)_{22}$ nanoparticles, inhibited the proliferation of tumors and decreased the induction of antioxidant defenses *in vivo* [18,19]. We have also determined that intraperitoneally injected $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles efficiently inhibited the growth of hepatoma cells implanted into the legs of mice and that inhibition of tumor growth involved reduction in tumor-induced oxidative stress rather than direct cytotoxicity to tumor cells [19]. However, the molecular mechanisms underlying these protective effects are still unclear.

It has been shown that a number of fullerenes, fullerenols, and endohedral metallofullerenols are capable of reacting with and/or scavenging free radicals [15,16,20]. However, much less is known about the protective role of Gd@$C_{82}(OH)_{22}$ nanoparticles. It has not been determined if the ROS-scavenging capability of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles is higher than the other functionalized fullerenes (e.g., functionalized C_{60}-fullerenols and C_{60}-carboxyfullerenes). In this study we employ the electron spin resonance (ESR) spin trap technique to provide direct *in vitro* evidence that Gd@$C_{82}(OH)_{22}$, a fullerenol ($C_{60}(OH)_{22}$), and a carboxyfullerene ($C_{60}(C(COOH)_2)_2$), can efficiently scavenge different types of free radicals and inhibit lipid peroxidation. Both ROS, i.e. superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2$), and the stable, nitrogen-centered free radical, $DPPH^{\bullet}$ were intercepted by these fullerene derivatives. Using human lung adenocarcinoma A549 cells or rat brain capillary endothelial cells (rBCECs), we further demonstrate that these fullerene derivatives reduce H_2O_2-induced cytotoxicity, free radical formation and mitochondrial damage.

2. Materials and methods

2.1. Preparation and characterization of water-soluble fullerene derivatives

Highly purified hydroxylated and malonic acid-substituted derivatives of C_{60} were prepared by previously published methods [21,22]. Although the diameter of an isolated C_{60} molecule is about 0.7 nm, fullerene derivatives readily aggregate and form nanoparticles in aqueous solution. Additional physical and chemical properties of $C_{60}(OH)_{22}$ and $C_{60}(C(COOH)_2)_2$ can be found in the previous reports [21,22]. The synthesis and characterization of Gd@$C_{82}(OH)_{22}$ have been previously described [18]. The nanoparticles' sizes were characterized in water using a field emission scanning electron microscope (FE-SEM, Hitachi S-4800, Japan). To investigate their particle sizes in the matrix used for ESR studies, we additionally measured their particle size distribution in phosphate buffered saline (PBS, 20 mM phosphate, 0.8% NaCl, pH 7.4) using dynamic light scattering (DLS) (Nano ZS90, Malvern). The DLS data were in agreement with those obtained by SEM (data not shown).

The fullerene derivatives used for ESR detection were prepared according to the experimental requirements (see materials and methods below). For cell experiments, nanoparticles of fullerene derivatives were diluted as needed with PBS prior to use. We further compared the spectra of different fullerenes, which were recorded between 200 nm and 600 nm using a UV–Vis spectrophotometer (CARY 100 Bio, Varian, Inc., USA) at room temperature of 20–25 °C.

2.2. Reagents

Hydrogen peroxide (H_2O_2), xanthine, diethylenetriaminepentaacetic acid (DTPA), 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) and 2,2,6,6-tetramethyl-4-piperidone (TEMP) were purchased from Sigma Aldrich (St. Louis, MO) 5-tert-Butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO) and 5-diethoxyphosphoryl-5-methyl-I-pyrroline N-oxide (DEPMPO) were supplied by Oxis International (Portland, OR). Basic endothelial cell growth factor (bECGF) and xanthine oxidase were obtained from Roche Applied Science (Indianapolis, IN). Egg phosphatidylcholine was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA). All reagents used in cell culture were obtained from HyClone Co. (Logan, UT) unless otherwise stated. Fluorescent probes JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) and CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) were purchased from Invitrogen Co. (Molecular Probes, Eugene, OR). All other reagents used were at least of analytical grade.

2.3. Cell cultures

rBCECs were prepared following a modified protocol of Deli et al. [23]. In brief, four Wistar rats (85–90 g) were sacrificed by cervical dislocation, the forebrains were collected, meninges were removed and the grey matter was minced and digested with type II collagenase. The cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM) and centrifuged at 1500 rpm for 5 min. After three cycles of suspension and centrifugation, the pellet containing capillary endothelial cells was collected and cultured in DMEM medium with 20% fetal bovine serum (FBS), supplemented with 10 U/mL heparin (Sigma–Aldrich Co., St. Louis, MO), 100 U/mL penicillin–streptomycin solution and 150 μg/mL endothelial cell growth factor (bECGF). All experiments used rBCECs cells in the third passage. A549 cells from the American Type Culture Collection (ATCC) (Manassas, VA) were maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/mL, penicillin–streptomycin) at 37 °C in 5% CO_2. Experiments were performed at least 3 times.

2.4. Cell viability assessment

The activity of mitochondrial dehydrogenase, a critical measure of mitochondrial function, and cellular toxicity, was determined according to a protocol previously described. Briefly, rBCECs or A549 cells at 90% confluence were cultured with different concentrations of fullerene derivative nanoparticles (10, 50, 100 μM) for 24 h. The medium was then replaced with fresh medium containing 50 μM H_2O_2 (Chemical Reagents Co., Beijing). After treatment with H_2O_2 for 2 h, cells were washed three times with PBS. The mitochondria's ability to reduce a tetrazolium salt to a formazan dye was used to assess mitochondrial dehydrogenase activity. Briefly, a certain volume of solution containing tetrazolium salt (available in the CCK-8 Kit from Dojindo Laboratories, Japan) was added to the culture medium. After incubation for 1.5 h at 37 °C, the absorbance at 450 nm was read using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA). Measurement for each treatment was repeated in triplicate.

2.5. Measurement of mitochondrial membrane potential

The fluorescent potentiometric dye JC-1 is a cationic carbocyanine compound that accumulates in mitochondria and can be used to measure the mitochondrial membrane potential ($\Delta\Psi_m$). In intact cells, JC-1 accumulates in the mitochondria as aggregates and exhibits a fluorescence emission shift from green (~525 nm), the